Cytotoxic triterpenes from *Salvia buchananii* roots

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ABSTRACT

A pentacyclic triterpene, named salvibuchanic acid (**1**), together with five known compounds, were isolated from the roots of *Salvia buchananii* Hedge (Lamiaceae). The structural characterization of all compounds was performed by spectroscopic analyses, including 1D and 2D NMR, and HR-ESI-MS experiments. The lupane triterpene (**1**) and hyptadienic acid (**2**) were investigated for their potential cytotoxic activity on Jurkat, HeLa, and MCF7 cell lines. Both compounds showed an interesting antiproliferative activity with similar potency in all cell lines. By means of flow cytometric studies, hyptadienic acid (**2**) induced in HeLa cells a S cell cycle block, while **1** elicited both cytostatic and cytotoxic responses.

KEYWORDS*: Salvia buchananii*; pentacyclic triterpenes; salvibuchanic acid; phenolic derivatives; cytotoxic activity.

1. Introduction

Salvia is an important genus of the Lamiaceae family (formerly Labiatae). Over 900 species are widely distributed in different regions around the world such as the Mediterranean area, Central Asia, Africa, and America. Secondary metabolites produced by *Salvia* plants include monoterpenoids, diterpenoids, having mainly an abietane or clerodane skeleton, sesquiterpenoids, triterpenoids, flavonoids, and polyphenols (Wu et al. 2012). Aerial parts and roots of *Salvia* genus have been used in traditional medicine for thousand years in the treatment of different diseases. Diterpenoids and phenolic derivatives isolated from different species showed antioxidant, anticoagulant, cytoprotective, antihypertensive, anti-fibrotic, anti-ischemia-reperfusion injury, antiviral and antitumor activities (Li et al. 2013).

Salvia buchananii Hedge is a herbaceous perennial shrub, up to 50 cm high, with purple flowers and ovate-lanceolate to spathulate leaves. In a previous chemical study of *S. buchananii* aerial parts clerodane diterpenes, ursolic and oleanolic acids were isolated from the surface dichloromethane extract (Bisio et al. 2015). Moreover, the dichloromethane extract of *S. buchananii* fresh aerial parts was also investigated for its antioxidant (Giamperi et al. 2012) and antibacterial activities (Bisio et al. 2015). In the course of our investigation on Algerian *Salvia* species (Kabouche & Kabouche 2008; Lakhal et al. 2014), herein, we report the isolation and the structure characterization of one triterpene, named salvibuchanic acid (**1**) from *S. buchananii* roots (Figure 1), along with other five known natural compounds. Besides, salvibuchanic acid (**1**) and hyptadienic acid (**2**) have been evaluated for their cytotoxic activities in Jurkat, HeLa, and MCF7 cancer cell lines. Both compounds showed an interesting antiproliferative activity with similar potency in all cell lines. Further studies revealed that **2** induced in HeLa cells a S cell cycle block, while **1** elicited both cytostatic and cytotoxic responses.

2. Results and discussion

Chloroform and chloroform-methanol extracts of *S.buchananii* roots, were fractionated by flash column chromatography followed by RP-HPLC, leading to the isolation of one triterpene (**1**) and five known compounds, namely hyptadienic acid (**2**) (Rao et al., 1990; Wang et al. 2009), maslinic acid (Savona et al. 1983), caffeic acid methyl ester, caffeic acid (Saleem et al. 2004), and nepetoidin B (Nakanishi et al. 1990). The structures of all compounds were determined by combination of NMR and MS analyses and comparison of data with those reported in the literature.

Compound **1** was isolated as a white powder. The molecular formula was established to be $C_{30}H_{48}O_4$ by ^{13}C NMR and HR-ESI-MS analyses (*m/z* 495.3442 $[M+Na]^+$, calcd. 495.3450). The negative ESI-MS spectrum showed an adduct ion peak at *m/z* 517 $[M+HCOO]^-$, whereas in the MS^2 spectrum a prominent fragment at *m/z* 499 $[M+HCOO-18]^-$ was observed, due to the loss of a water molecule. The neutral loss of CO_2 from the parent ion (*m/z* at 473 $[M+HCOO-44]^-$) indicated the presence of one carboxyl moiety. The ^{13}C NMR spectrum of **1** (Table S1) exhibited thirty signals, which were assigned to one carbonyl group (δ 180.7), 1,1-disubstituted double bond (δ 109.0 and 151.8), one hemiacetal group (δ 94.5), and one hydroxymethylene (δ 71.6). These evidences suggested the presence of a pentacyclic triterpene, belonging to the lupane series (Yook et al. 2002). The ^1H NMR spectrum of **1** (Table S1) confirmed the presence of a structure with six methyl groups (δ 0.83, 0.95, 1.03, 1.05, 1.07, and 1.72), two olefinic protons at δ 4.71 and 4.90 (each 1H, br s). Assignments of all ^1H and ^{13}C NMR chemical shifts were ascertained on the basis of 1D total correlation spectroscopy (TOCSY), double-quantum filtred correlated spectroscopy (DQF-COSY), heteronuclear single quantum coherence (HSQC) analyses. The 1D-TOCSY experiments suggested the sequences: C-1—C-2, C-5—C-7, C-9—C-13, C-15—C-16, and C-18—C-22 confirming the presence of a pentacyclic system. A combination of HSQC and heteronuclear multiple bond coherence (HMBC) data led to establish the location of all substituents. The HMBC correlations between the proton signals at δ 1.72 (H-30) and the carbon resonances at δ 48.0 (C- 19), 109.0 (C-29), and 151.8 (C-20); δ 4.71 (H-29b) and 4.90 (H-29a) and δ 48.0 (C-19) and 151.8 (C-20), confirmed the position of the C-20/C-29 double bond. The signal at δ 1.65 (H-18) correlated with

carbon resonances at δ 48.0 (C-19), 57.4 (C-17), 151.8 (C-20), and 180.7 (C-28), while the proton signal at δ 1.46 (H-22b) showed long range correlations between signals at δ 31.1 (C-21), 50.0 (C-18), and 57.4 (C-17), confirming the location of carboxylic group at C-17. The connectivities between the proton signal at δ 2.14 (H-1a) and the carbon resonances at δ 41.7 (C-10), 48.4 (C-9), 61.4 (C-5) and 94.5 (C-2) substantiated the presence of hemiacetal group at C-2. The location of the epoxy group at C-2/C-3 position was verified by HMBC correlations between signal at δ 3.90 (H-3a) and the resonances at δ 39.7 (C-4), 61.4 (C-5), and 94.5 (C-2) (Figure S1). This compound was previously reported in a Chinese patent and its stereochemistry at C-2 was reported as β (Gao et al. 2015). However, the presence of the hemiacetal function at C-2 leads to an α/β equilibrium. Compound **1** was therefore identified as 2,3-*seco*-2,3-epoxy-2-hydroxylup-20(29)-en-28-oic acid and was named salvibuchanic acid.

In the frame of our project aimed to study the cytotoxic activity of plant terpenes, the antiproliferative activity of compounds **1** and **2** was evaluated in Jurkat, HeLa, and MCF7 cancer cell lines. Cells were exposed to increased concentrations of **1** and **2** and cell viability was evaluated at 48 h by MTT assay. Half maximal inhibitory concentration (IC_{50}) values, obtained from dose-response curves, are shown in Table 1. Compounds **1** and **2** showed an interesting antiproliferative activity with similar potency in all cell lines. In particular, HeLa resulted slight more susceptible than Jurkat and MCF7 cells. Cells were also treated to different concentrations of etoposide, as a positive control, and showed three different IC_{50} values (Table 1). Jurkat cells were most the sensitive to the action of etoposide, followed by HeLa cells, whereas the MCF7 cells were the most resistant. Furthermore, the cytotoxic potential of compounds **1** and **2** was evaluated in PBMC from healthy donors, chosen as the normal counterpart of leukemia- derived Jurkat cell line. Compounds **1** and **2** did not cause any significant reduction of the number of freshly isolated non-proliferating PBMC, at least in the range of cytotoxic doses in leukemia cells. The mechanism(s) underlying their antiproliferative effect was further investigated in HeLa cells. To investigate whether compounds **1** and **2** reduced cells number by affecting cell cycle progression and/or by inducing cell

death, HeLa cells were exposed for 48 h at concentrations close to their IC_{50} value, 30 and 50 µM for compound **1** and 15 and 35 µM for compound **2**, respectively; DNA content was evaluated by flow cytometry analysis of propidium iodide (PI) stained nuclei. As shown in Figure 2A, compound **1** caused an increase of cell population in S phase. Moreover, an increase of cells with sub G_0/G_1 DNA content was observed, thus indicating the onset of apoptotic events. Compound **2** induced a robust and dose-dependent S block without any significant increase of hypodiploid cells (Figure 2B).

In conclusion, salvibuchanic acid (**1**), a lupane triterpene having an unusual seven-membered lactol ring in the A-ring is reported. The cytotoxic activity of salvibuchanic acid (**1**) and hyptadienic acid (**2**) was evaluated: these triterpenes may be worthy of further research as potential anticancer drugs.

3. Experimental

Salvibuchanic acid (**1**). White powder $[\alpha]_D$ = +17 (c 0.1, MeOH); HR-ESI-MS: *m/z* 495.3442 $[M+Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450), 477.34 $[M+Na-18]^+$; ESI-MS: *m/z* 517 $[M+HCOO]^-$, 499 $[M+HCOO-18]^+$, 473 $[M+HCOO-44]^-$; ^{1}H NMR (600 MHz, CD_3OD) δ: 0.83 (3H, s, Me-23), 0.88 (1H, dd, *J* = 11.8, 6.0 Hz, H-5), 0.95 (3H, s, Me-24), 1.03 (3H, s, Me-27), 1.05 (3H, s, Me-25), 1.07 (3H, s, Me-26), 1.21 (1H, m, H-15b), 1.30 (1H, dd, *J* = 9.5, 4.2 Hz, H-9), 1.37 (1H, overlapped, H-1b), 1.38 (1H, overlapped, H-21b), 1.40 (1H, overlapped, H-16b), 1.44 (1H, overlapped, H-7b), 1.46 (1H, overlapped, H-22b), 1.54 (1H, overlapped, H-15a), 1.55 (1H, overlapped, H-6b), 1.57 (1H, overlapped, H-6a), 1.62 (1H, overlapped, H-12b), 1.64 (1H, overlapped, H-12a), 1.65 (1H, br d, *J* = 11.5 Hz, H-18), 1.72 (1H, s, Me-30), 1.93 (1H, m, H-22a), 1.98 (1H, overlapped, H-21a), 2.14 (1H, dd, *J* = 13.0, 1.5 Hz, H-1a), 2.28 (1H, m, H-7a), 2.42 (1H, dd, *J* = 11.4, 4.0 Hz, H-13), 3.04 (1H, d, *J* = 11.8 Hz, H-3b), 3.09 (1H, ddd, *J* = 16.0, 11.5, 4.6 Hz, H-19), 3.90 (1H, d, *J* = 11.8 Hz, H-3a), 4.71 (1H, br s, H-29b), 4.90 (1H, br s, H-29a), 5.00 (1H, dd, *J* = 11.0, 5.5 Hz, H-2); ^{13}C NMR (600 MHz, CD_3OD) δ: 14.6 (C-27), 15.0 (C-25), 17.0 (C-26), 19.1 (C-30), 20.7 (C-24), 22.0 (C-11), 23.0 (C-6), 26.8 (C-12), 27.6 (C-23), 30.5 (C-15), 31.0 (C-16),

31.1 (C-21), 33.1 (C-7), 38.0 (C-22), 39.6 (C-13), 39.7 (C-4 and C-8), 41.7 (C-10), 43.4 (C-14), 45.8 (C-1), 48.0 (C-19), 48.4 (C-9), 50.0 (C-18), 57.4 (C-17), 61.4 (C-5), 71.6 (C-3), 94.5 (C-2), 109.0 (C-29), 151.8 (C-20), 180.7 (C-28).

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Bisio A, Schito AM, Parrichi A, Mele G, Romussi G, Malafronte N, Oliva P, De Tommasi N. 2015. Antibacterial activity of constituents from *Salvia buchananii* Hedge. Phytochem Lett. 14:170-177.

Gao P, Mou X, Liu X, Jiang F, Zhao H, Wang Y, Zhang Z. 2015. Triterpene compound with inhibiting activity on acetylcholinesterase and preparation method. Faming Zhuanli Shenqing CN 104788525 A 20150722.

Giamperi L, Bucchini A, Bisio A, Giacomelli E, Romussi G, Ricci D. 2012. Total phenolic content and antioxidant activity of *Salvia spp. exudates*. Nat Prod Commun. 7:201-202.

Kabouche A, Kabouche Z, 2008. Bioactives diterpenoids of *Salvia* species. Nat Prod Chem. 35:753-833.

Lakhal H, Kabouche A, Alabdul Magid A, Voutquenne-Nazabadioko L, Harakat D, Kabouche Z. 2014. Triterpenoids from *Salvia argentea* var. *aurasiaca* (Pomel) Batt. & Trab. and their chemotaxonomic significance. Phytochemistry. 102:145-151.

Li M, Li Q, Zhang C, Zhang N, Cui Z, Huang L, Xiao P. 2013. An ethnopharmacological investigation of medicinal *Salvia* plants (Lamiaceae) in China. Acta Pharm Sin B. 3:273-280.

Nakanishi T, Nishi M, Inada A, Obata H, Tanabe N, Abe S, Wakashiro M. 1990. Two new potent inhibitors of xanthine oxidase from leaves of *Perilla frutescens* Britton var. *acuta* Kudo. Chem Pharm Bull. 38:1772-1774.

Rao KVR, Rao LJN, Rao NSP. 1990. An A-ring contracted triterpenoid from *Hyptis Suaveolens*. Phytochemistry. 29:1326-1329.

Saleem M, Kim HJ, Jin C, Lee YS. 2004. Antioxidant caffeic acid derivatives from leaves of *Parthenocissus tricuspidata*. Arch Pharm Res. 27:300-304.

Savona G, Bruno M. 1983. Terpenoids of Cultivated *Salvia canariensis*. J Nat Prod. 46:593-594.

Wang F, Li XM, Liu JK. 2009. New terpenoids from *Isodon sculponeata*. Chem Pharm Bull. 57:525-527.

Wu YB, Ni ZY, Shi QW, Dong M, Kiyota H, Gu YC, Cong B. 2012. Constituents from *Salvia* species and their biological activities. Chem Rev. 112:5967-6026.

Yook CS, Liu XQ, Chang SY, Park SY, Nohara T. 2002. Lupane-Triterpene glycosides from the leaves of *Acanthopanax gracilistylus*. Chem Pharm Bull. 50:1383-1385.

Table 1. IC_{50} (μM) values of compounds **1** and **2** against three cancer cell lines[a].

Compounds	Jurkat	HeLa	MCF7
1	38 ± 0.9	40 ± 2.1	70 ± 3.1
2	30 ± 13	25 ± 1.5	55 ± 2.3
Etoposide	2.5 ± 0.8	4 ± 1.1	12 ± 2.2

[a] Mean values ± SD from three experiments done in quadruplicate.

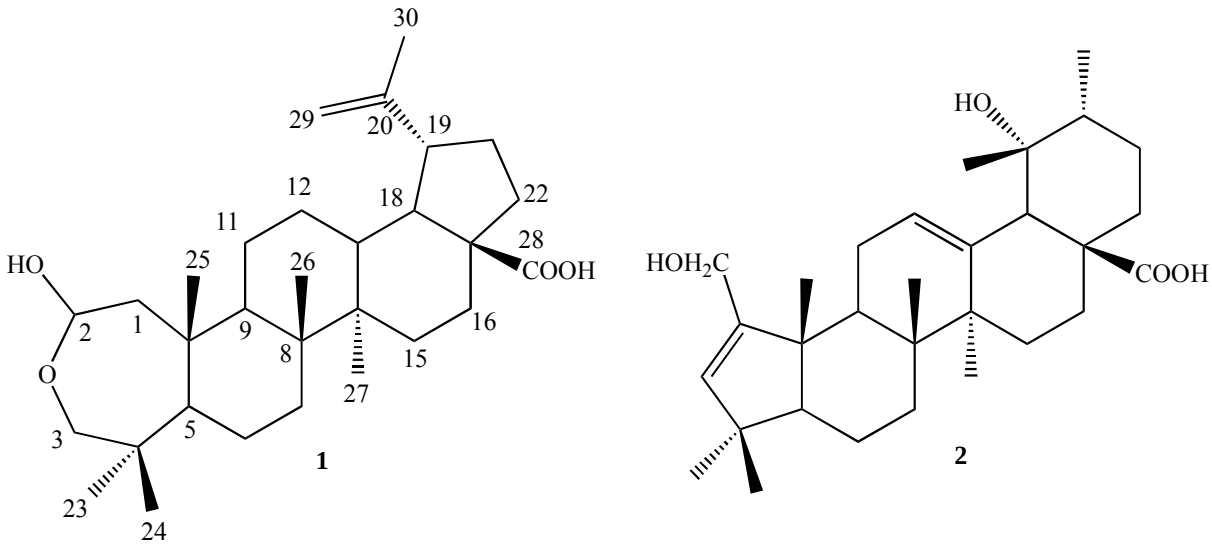

Figure 1. Structures of compounds **1** and **2**.

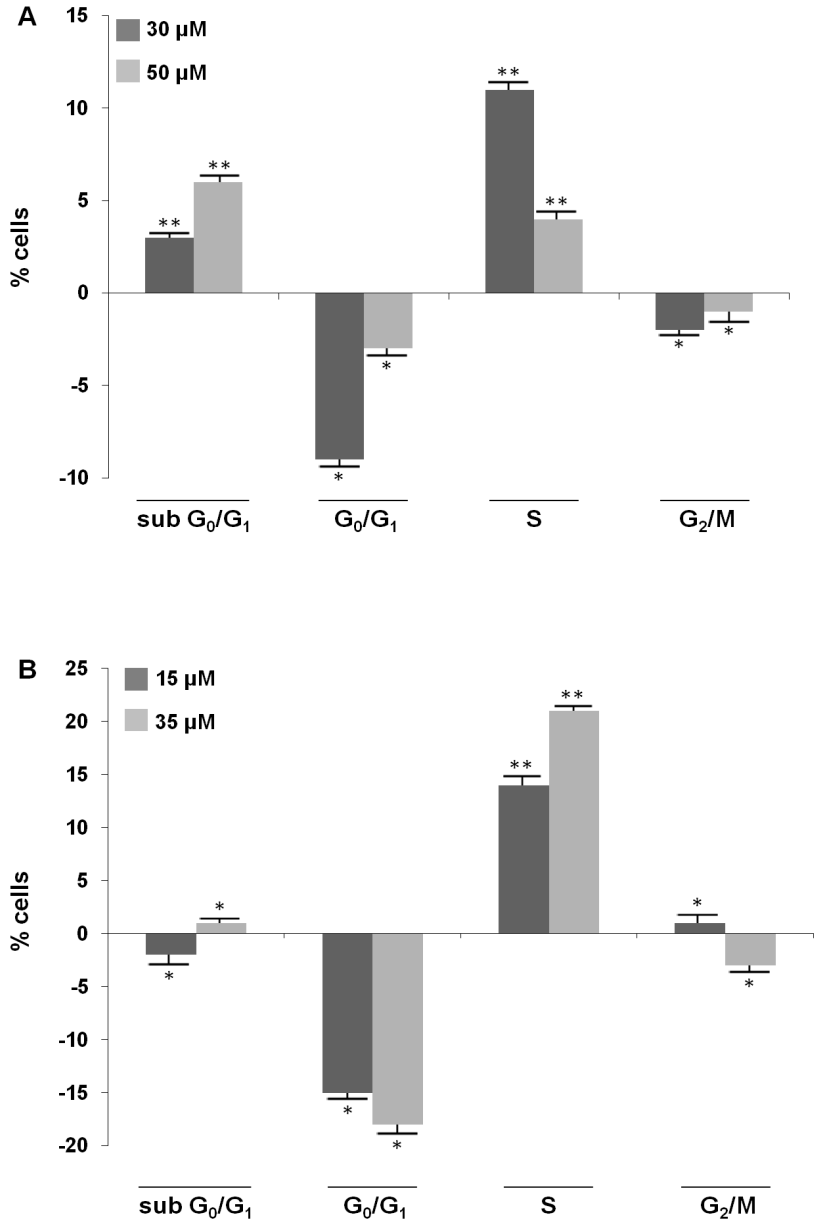

Figure 2. Effect of compounds **1** and **2** on proliferation of HeLa cells. Flow cytometric evaluation of DNA content in HeLa cells exposed for 48 h to **1** and **2** or vehicle alone (controls). (**A**) HeLa cells exposed to 30 and 50 µM compound **1**; (**B**) HeLa cells exposed to 15 and 35 µM compound **2**. Data are presented as increase⁄decrease in percentages of treated cells with a specific DNA content, in respect to control values (control cells, subG_0⁄G_1, ≤2%; G_0⁄G_1, 53 ± 1.8%; S, 37 ± 2.1%; G_2⁄M, 12 ± 0.9%). All results are mean values ± SD from at least three experiments performed in duplicate (*$p < 0.05$, **$p < 0.001$).